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Applications of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

Analysis of feed grains, intravenous solutions and glycoproteins

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ABSTRACT

Primary and secondary amines are rapidly labelled by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to form highly fluorescent asymmetric urea derivatives which are readily amenable to analysis by liquid chromatography. Derivatization consists of a simple, one-step procedure, and the resulting labelled amines can be analyzed without further cleanup. The adducts are extremely stable with no discernible loss in response after storage for one week at room temperature, making the reagent an ideal candidate for pre-column amino acid analysis. Chromatographic methods for protein hydrolysates have been developed for the analysis of samples containing many unusual amino acids including a number of cysteine derivatives, collagen hydrolysates containing hydroxyproline and hydroxylysine, performic acid oxidized samples and glycoprotein hydrolysates containing glucosamine and galactosamine. Samples with potentially interfering matrix components such as hydrolyzed feed grains and intravenous solutions are readily analyzed and are quantified with average per cent relative standard deviations in the 1–2% range. Comparative data on these samples are in good agreement with either ion-exchange amino acid analysis or label information.

INTRODUCTION

Development of versatile, high-performance systems for amino acid analysis continues to be a subject of considerable interest [1–4]. We have recently developed a new method for amino acid analysis based on pre-column derivatization with the novel reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) that provides dramatic improvements over existing methodologies [5]. Documented advantages include a rapid, simple derivatization protocol, excellent response linearity over at least two orders of magnitude, formation of highly stable urea derivatives, and detection limits below one picomole. The derivatization reaction has several key features that simplify analysis: (1) formation of amine or amino acid derivatives is extremely rapid, occurring within seconds, (2) excess reagent is hydrolyzed to 6-aminoquinoline (AMQ) in less than 2 min, thus preventing any unwanted side reactions and (3) fluorescence emission maxima of AMQ and AQC-derivatized amines

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are approximately 100 nm apart, allowing for selective detection of the desired analytes without significant reagent interference. Further studies with peptide and protein hydrolysates have shown the derivatization chemistry to be reproducible and provide excellent compositional analyses [6,7]. These results are comparable to the traditional ion-exchange method of analysis [8,9] with typical analyses providing average compositional errors of 3–8% with sample quantities ranging from 0.2–5.0 μg, approximately an order of magnitude smaller than that used for the traditional procedure.

Amino acid analysis using reversed-phase chromatography has been often cited for its versatility and flexibility [1,2,10–12], but there have been reports of problems due to interference by the sample matrix with samples such as hydrolyzed grains or with samples containing buffer salts. We have concentrated our most recent efforts on extending the capabilities of AQC-amino acid analysis from compositional analysis of peptides and proteins to a number of different sample types requiring modified sample preparation, and/or new chromatographic conditions for complete analysis. This report will describe the analysis of hydrolyzed grain samples, hydrolyzed food or feed samples including collagen-containing samples, glycoproteins and intravenous solutions. Chromatographic conditions for many common amino acids not present in standard hydrolysate samples have also been developed, and the key chromatographic parameters used for separation optimization will be described.

MATERIALS AND METHODS

Chemicals

AQC (Waters AccQ·Fluor reagent) and borate buffer were obtained as a kit from Millipore (Milford, MA, USA). Eluent A concentrate was also from Millipore. Water was supplied by a Milli-Q system purchased from Millipore (Bedford, MA, USA). Sodium acetate trihydrate (HPLC grade) and disodium ethylenediaminetetraacetic acid were from Baker (Phillipsburg, PA, USA); triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI, USA). Amino acid standards were from Pierce (Rockford, IL, USA) or Sigma (St. Louis, MO, USA); proteins were purchased from Sigma. Intravenous solutions were from Laboratories Don Baxter (Freeamine III; Trieste, Italy) and Soluzione di L-Aminoacidi Selettivi All' 8%, Bieffe Medital (Modena, Italy); grain samples were from a collaborative study organized by DeGussa Inc.

Chromatography instrumentation

The HPLC system consisted of a 625 LC solvent delivery system equipped with a column heater, a 717 plus autosampler with heater/chiller accessory, a 470 scanning fluorescence detector, and a 486 variable-wavelength detector (all Waters components, Millipore, Milford, MA, USA). A Waters Millennium 2010 workstation was used to control system operation and collect and analyze data.

Protein hydrolysis

Samples dissolved in water or dilute HCl (0.1–5.0 μg) were pipetted into 50 × 6 mm test tubes, vacuum dried and batchwise hydrolyzed *in vacuo* with 200 μl of constant-boiling HCl with *ca.* 0.5 mg crystalline phenol as a scavenger [13]. The samples were heated at 114°C for 20 h, cooled to room temperature and dried to remove excess HCl. Amino acids were reconstituted with 20 μl of 20 m*M* HCl and derivatized as described below.

Intravenous solutions

Samples were diluted 200-fold with water and α-aminobutyric acid was added as an internal standard to give a final concentration of 0.1 m*M*. The diluted sample was derivatized as described below.

Grain samples

Feed grains were milled to a uniform size and 296 mg weighed into a screw top test tube. A 50-ml volume of 6 *M* HCl was added and the sample sealed after flushing with nitrogen for 5 min. Some samples were oxidized with performic acid prior to hydrolysis according to previously published methods [14]. After hydrolysis 20 ml of internal standard Nle (0.19 mg/ml) was

added, the volume reduced to 2 ml, and then 50 ml of 0.2 *M* sodium citrate pH 2.20 was added.

Sample derivatization

In a typical analysis, 10–20 μl of sample were buffered with 0.2 *M* sodium borate, pH 8.8 containing 5 m*M* disodium EDTA (total volume 80 μl). The derivatization reaction was then initiated by the addition of 20 μl of AQC solution (3 mg/ml in acetonitrile). Grain samples were diluted to 200 μl with borate and derivatized with 50 μl of reagent. The reaction was terminated within 2 min due to reagent hydrolysis [5] and reversal of tyrosine phenol modification to the unmodified form accelerated by heating at 55°C for 10 min. The heating step was occasionally automated with a heated 717 plus autosampler at 40°C for 90 min. From this sample, 5–20 μl were then analyzed by HPLC.

Chromatographic analysis

Separations were carried out on a 150 × 4.6 mm AccQ · Tag C_{18} reversed-phase column (Millipore). Two eluent systems (eluents 1 and 2) used a concentrated eluent formulation before final dilution. Concentrate was made by dissolving 190.4 g sodium acetate trihydrate (Baker HPLC grade) in 1 l of water, adding 23.7 ml of triethylamine and titrating the solution with 50% phosphoric acid. The concentrate contained 10 ml of a 1 g/l solution of disodium EDTA and 0.1% sodium azide as a preservative. Eluent 1 used concentrate at pH 5.02 and working eluent was made with 100 ml of concentrate and 1000 ml of water. Eluent 2 used concentrate at pH 5.10 and contained 100 ml of concentrate and 800 ml of water. Eluent 3 was a ready-to-use formulation containing 19.04 g sodium acetate and 970 μl of TEA titrated to pH 5.80. EDTA and azide were added at one-tenth the concentration of the concentrate described above. Eluent B was acetonitrile and eluent C was water.

RESULTS AND DISCUSSION

Sample derivatization

Samples buffered at pH 8.8 are rapidly derivatized in a single step by the addition of the AQC solution. Within 2 min excess reagent is hydrolyzed to produce 6-aminoquinoline, N-hydroxysuccinimide and CO_2 [5]. The derivatization reaction for amines is shown in Fig. 1. A highly favorable blue shift in the fluorescence emission maximum of the AQC-labelled amino acids *versus* the hydrolysis product AMQ [5] enables direct injection of the derivatized mixture without any further sample workup.

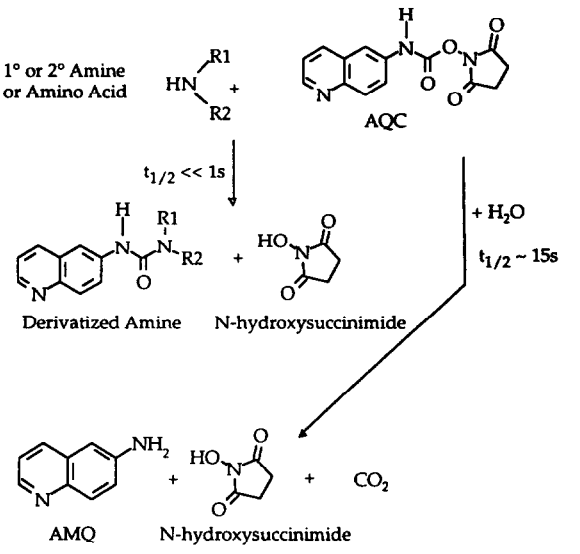

Fig. 1. Derivatization of primary (1°) and secondary (2°) amines with AQC. Also shown is the hydrolysis reaction of excess reagent.

Analysis of intravenous (i.v.) solutions

Chromatography of i.v. solutions employed eluent 1 and a multi-step gradient profile given in Table I (system 1). A typical analysis is shown in Fig. 2 where detection was accomplished by both fluorescence and UV at 254 nm to facilitate tryptophan detection. Analytical reproducibility is a priority concern with a pharmaceutical sample such as an i.v. solution. Table II presents a summary of 10 replicate derivatizations of an i.v. solution and a comparison of the recoveries with the sample label information. Data were generated in two laboratories using AQC derivatization, one system using both fluorescence and UV detection, the second being equipped

TABLE I

GRADIENT TABLES FOR THREE CHROMATOGRAPHY SYSTEMS

Eluent A is described in the text, eluent B was acetonitrile, and eluent C was water. Separation used only eluents A and B, with the water used for column flushing at the end of each analysis using 60% acetonitrile in water. After washing the column for 4 min it was re-equilibrated in 100% eluent A for 9 min. The total run time for system 1 was 45 min, 50 min for system 2 and 63 min for system 3. The gradient profile for each step was either linear (L) or a step (S) segment. * is initial conditions.

System 1			System 2			System 3		
Time (min)	MeCN (%)	Curve	Time (min)	MeCN (%)	Curve	Time (min)	MeCN (%)	Curve
0	0	*	0	0	*	0	0	*
0.5	1	S	0.5	1	S	1	1	S
18	5	L	16	3	L	16	3	L
19	9	L	23	8	L	25	6	L
29.5	18	L	36	19	L	35	14	L
33	18	L	38	19	L	40	14	L
						50	18	L

only with a fluorescence detector. Recoveries were extremely reproducible with average relative standard deviations (R.S.D.s) of approximately 1.4%. Interlaboratory comparison indicated that the method was capable of generating similar results with two completely different laboratory systems, and completely independent sample preparation. All data were generated by fluorescence detection except for the analysis of Trp, whose fluorescence response is weak due to internal quenching, which used UV detection.

Analysis of grain samples

Oxidation with performic acid produces two additional amino acids, methionine sulfone and cysteic acid, from methionine and cyst(e)ine respectively. Complete resolution of these derivatives and the normal hydrolysate amino acids

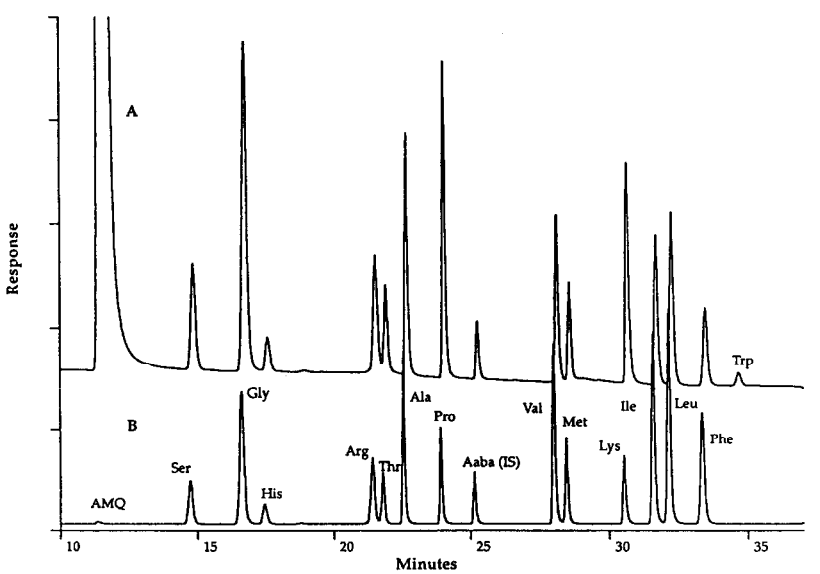

Fig. 2. Analysis of the Baxter Freeamine III intravenous solution. Sample preparation and chromatography are described in the text and Table I. The column was thermostatted at 37°C. Detection was by UV at 254 nm, full scale = 0.2 AU (A) and by fluorescence with excitation at 250 nm and emission at 395 nm, full scale = 2 V (B).

TABLE II

ANALYSIS OF INTRAVENOUS SOLUTIONS

ND = Not determined.

Amino acid	Baxter i.v. solution			Bieffe i.v. solution		
	Label value (mg/100 ml)	Laboratory 1 (% Label value)[a]	Laboratory 2 (% Label value)[b]	Label value (mg/100 ml)	Laboratory 1 (% Label value)[a]	Laboratory 2 (% Label value)[b]
Ser	500	97.2	104.4	430	95.7	99.5
Gly	1190	95.3	101.8	950	103.6	100.5
His	240	115.8	106.6	298	105.8	100.8
Arg	810	103.5	108.4	780	103.3	106.4
Thr	340	109.1	107.4	580	108.3	104.6
Ala	600	100.7	103.4	550	104.6	103.3
Pro	950	104.6	104.4	580	105.4	101.9
Val	560	106.4	104.7	1065	108.4	101.0
Met	450	108.7	106.0	50	144.8 (114.8)[d]	82.2
Lys	870	102.0	106.3	760	101.7	103.7
Ile	590	106.4	104.3	709	106.4	102.7
Leu	770	104.9	103.8	1184	107.3	103.6
Phe	480	109.2	102.4	27	118.1	99.6
Trp	130	100.7[c]	64	15	ND	ND

[a] Average of 10 injections.
[b] Average of 3 injections.
[c] Concentration based on UV data.
[d] Met data reported based on peak area and peak height measurements (in parentheses).

required modification of the original conditions. The key peak pair was alanine and methionine sulfone which could be resolved through a combination of gradient, eluent and column temperature changes. Thus, increasing the pH and eluent ionic strength slightly with eluent 2 and decreasing the column temperature from 37 to 31°C yielded the separation shown in Fig. 3. The gradient profile (system 2) is given in Table I. These chromatographic conditions have also proved useful for the analysis of collagen-type samples containing hydroxyproline and hydroxylysine (Fig. 4).

Replicate analyses of hydrolyzed grain samples exhibit highly reproducible amino acid yields (average R.S.D. = 1.1%) which are well-correlated with results obtained by ion-exchange amino acid analysis with post-column derivatization by *ortho*-phthalaldehyde (Table III). Comparative data in Table III have been normalized to the recovery of leucine, with the data from the AQC-derivatized samples then expressed as a percentage of the ion-exchange (IEX) results. The formula below was used to calculate the yield determined by AQC derivatization relative to the ion-exchange analysis:

$$\text{Per cent yield amino acid} = \frac{\text{AQC yield amino acid/AQC yield Leu}}{\text{IEX yield amino acid/IEX yield Leu}} \cdot 100\%$$

Glycoprotein analysis

The analyses of derivatized glycoprotein hydrolysates are shown in Fig. 5. Different classes of glycoproteins are represented by lactoferrin, α_1-acid glycoprotein and fetuin, which contain N-linked, O-linked and both types of oligosaccharides, respectively. The significance of these different classes with respect to amino acid analysis is the presence of either N-acetyl galactosamine ($GalNH_2$) in the O-linked glycoproteins or N-acetyl glucosamine ($GlcNH_2$) in the N-linked oligosaccharides which upon hydrolysis yield their respective hexosamines. These form

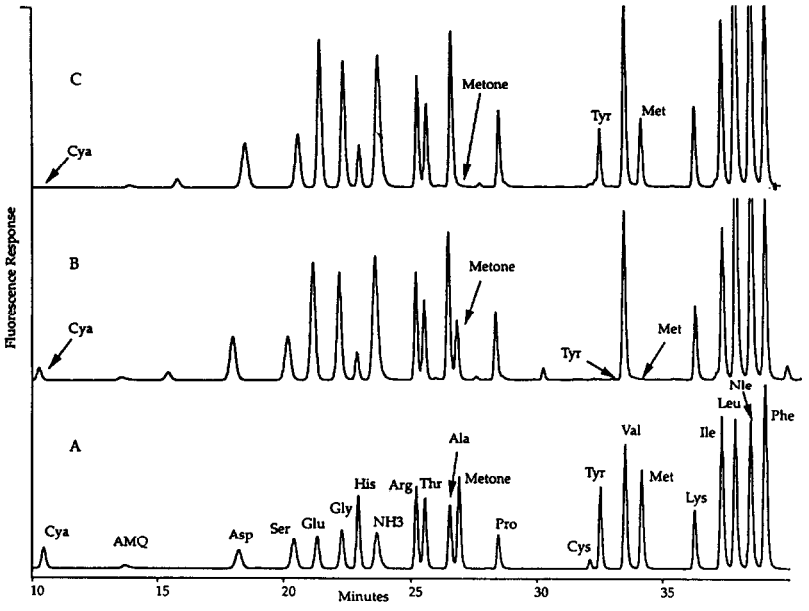

Fig. 3. Analysis of a standard containing cysteic acid (Cya) and methionine sulfone (Metone) (A), and a hydrolyzed grain sample with (B) and without (C) performic acid oxidation prior to hydrolysis. Eluent and gradient conditions are described in the text and Table I. The column was thermostatted at 31°C. Norleucine (Nle) was used as an internal standard.

stable derivatives with AQC which must be resolved from the normally present amino acids to allow for good quantitation. It is worth noting that such hydrolysates should not be used to quantitate the amino sugars as recoveries after hydrolysis are significantly less than quantitative [15].

The chromatography using eluent 3 and the

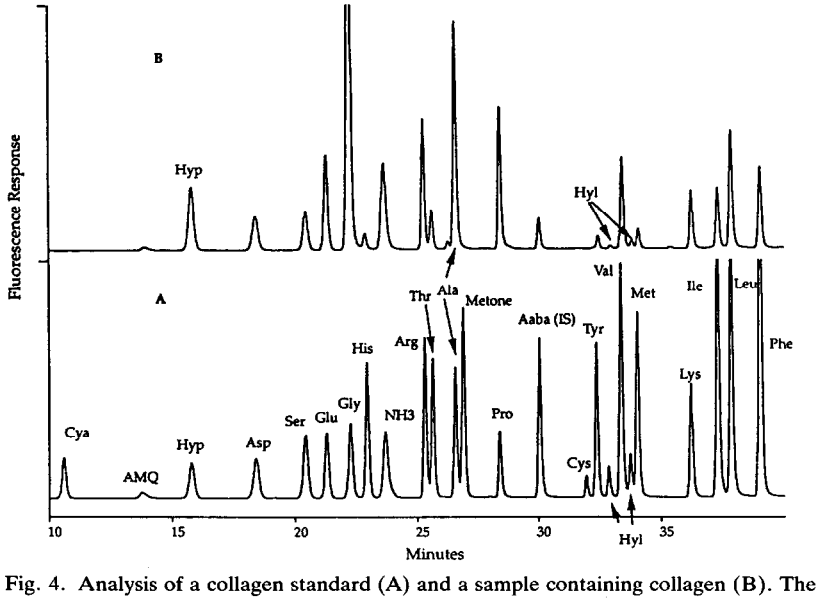

Fig. 4. Analysis of a collagen standard (A) and a sample containing collagen (B). The internal standard was α-aminobutyric acid (Aaba). Conditions as in Fig. 3.

TABLE III

REPRODUCIBILITY DATA FOR FEED GRAIN SAMPLE

	Retention time reproducibility		Amount reproducibility R.S.D. (%) ($n = 10$)[a]	Comparison with ion-exchange[b]
	n	R.S.D. (%)		
Cya	10	1.29	1.31	1.33
Asp	20	0.92	1.86	0.98
Ser	20	0.61	1.72	0.93
Glu	20	0.46	1.70	0.96
Gly	20	0.37	1.72	0.87
His	20	0.32	1.75	1.01
Arg	20	0.25	1.27	1.00
Thr	20	0.24	1.44	0.98
Ala	20	0.23	0.88	1.00
Metso	10	0.22	1.21	0.86
Pro	20	0.16	0.88	
Tyr	10	0.07	1.75	1.19
Val	20	0.10	0.62	0.96
Lys	20	0.09	2.62	1.01
Ile	20	0.09	0.42	1.05
Leu	20	0.09	0.20	1.00
Phe	20	0.10	0.28	1.06
Average R.S.D. (%)		0.33	1.27	

[a] Data for Cya and Metone used performic acid oxidized samples, other data were from unoxidized samples.

[b] Ion-exchange data were kindly provided by Mike Kennedy, Cargill, Inc. (Minnetonka, MN, USA). See the text for the calculation method used.

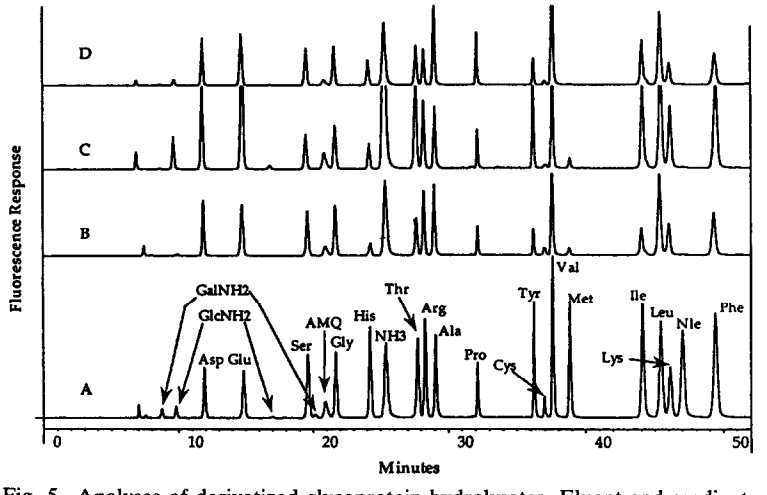

Fig. 5. Analyses of derivatized glycoprotein hydrolysates. Eluent and gradient conditions are described in the text and Table I. The column was thermostatted at 37°C. The samples are standard mixture with glucosamine and galactosamine (A), lactoferrin (B), α_1-acid glycoprotein (C) and fetuin (D).

gradient described in Table I (system 3) resulted in some significant changes in relative retention of key amino acids. At the higher pH of the mobile phase, the acidic amino acids Asp and Glu are retained much less relative to other amino acid derivatives as the side chain becomes deprotonated. Conversely, AMQ has much greater retention and elutes between Ser and Gly. This would make UV detection impractical as the reagent peak would interfere with Gly quantitation, but poor fluorescence emission for AMQ at the amino acid derivative emission maximum at 395 nm makes it feasible to use conditions under which AMQ elutes between components of interest. Other changes in elution order observed include reversal of Arg and Thr, Cys and Tyr, and elution of Lys after Ile and Leu.

Derivatization of standard solutions of either $GalNH_2$ or $GlcNH_2$ resulted in the appearance of a major peak and a minor peak. This complicated the analysis of $GlcNH_2$ containing samples as the smaller peak interfered with Ser if the samples were analyzed with the lower eluent pH separation system used for either i.v. solutions or grain samples. However, glycoproteins with N-linked carbohydrates could be analyzed with the system using eluent at pH 5.02 as both the major and minor peaks were resolved from the other amino acid derivatives (data not shown). One possible source of the multiple derivatives for the aminosugars is resolution of anomeric forms. This hypothesis is consistent with previous studies on glycoprotein analysis using OPA derivatization [16].

Compositional analysis of the glycoprotein lactoferrin is shown in Table IV. Error data were calculated according to the procedure described by Strydom *et al.* [3] and provide an unbiased measure of compositional accuracy. With highly purified proteins and sample amounts of 2–5 μg, typical analytical procedures give average errors of *ca.* 10% [3]. The data in Table IV were obtained from *ca.* 1.6 μg of hydrolyzed protein and give an excellent analysis with an average error of 7.1%.

Analysis of alkylated cysteine derivatives

Because cysteine and cystine are not quantitatively recovered after standard hydrolysis proce-

TABLE IV

COMPOSITIONAL ANALYSIS OF LACTOFERRIN

The hydrolyzed sample contained approximately 1.6 μg; 5% was analyzed by HPLC.

Amino acid	Literature composition (residues/mol)	Calculated composition (residues/mol)	Error (%)
Asp	77	74.42	3.35
Glu	71	77.74	9.50
Ser	50	48.95	2.09
Gly	46	54.17	17.77
His	9	8.81	2.06
Thr	31	31.75	2.43
Arg	46	45.56	0.95
Ala	63	60.30	4.29
Pro	35	37.49	7.11
Tyr	20	20.57	2.85
Val	49	46.49	5.12
Met	6	4.56	24.05
Ile	16	18.17	13.57
Leu	61	57.65	5.49
Lys	46	43.94	4.49
Phe	31	28.64	7.61
Average error (%)			7.05

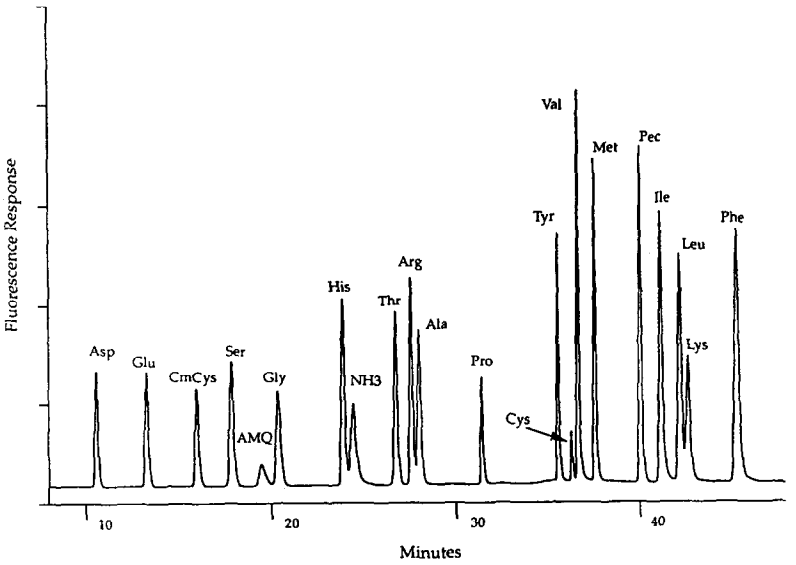

Fig. 6. Analysis of a hydrolysate standard plus the alkylated cysteine derivatives carboxymethyl cysteine (CmCys) and pyridylethyl cysteine (Pec). Conditions as in Fig. 5.

dures, chemical modification of these amino acids to more stable derivatives is usually performed. Previously, we have shown analysis of cyst(e)ine modified with the disulfide interchange reagent dithiodipropionic acid, and in this paper we have described the analysis of grain samples oxidized with performic acid. These are both destructive techniques in that the protein cannot be used for subsequent analytical procedures such as peptide mapping or N-terminal sequence analysis after the modification. Alkylation of the sulfhydryl group is an established procedure for non-destructive cyst(e)ine modification that yields acid-stable derivatives. Two of the more common reagents are iodoacetic acid [17] and 4-vinyl pyridine [18] which react with cysteine to form carboxymethyl cysteine and pyridylethyl cysteine, respectively. Using the conditions described for the analysis of glycoproteins (gradient system 3 and eluent 3), the AQC derivatives of these modified amino acids are easily resolved from the other amino acids allowing for quantitation of all the amino acid components (Fig. 6).

CONCLUSIONS

Derivatization of amino acids with AQC is a simple, highly reproducible, accurate procedure that is easily adapted for a wide variety of samples including feed grains, intravenous solutions, and glycoproteins. Derivative yields are essentially unaffected by the presence of metallic salts and high concentrations of carbohydrates present in these samples. Flexible chromatographic systems have allowed the development of conditions that resolve a number of amino acids in addition to those present in standard protein hydrolysates. This versatile reagent will undoubtedly be useful in a number of important amino acid analysis applications. Recent work in our laboratory suggests that the range of applications can be routinely expanded to a number of interesting target molecules such as amine drugs and peptides [19].

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